Distinguishing Features of Anti-β2 Glycoprotein I Antibodies between Patients with Leprosy and the Antiphospholipid Syndrome

J. Arvieux1, Y. Renaudineau1, I. Mane2, R. Perraut3, S. A. Krills4, P. Youinou1

1Laboratoire d’Immunologie, Institut de Synergie des Sciences et de la Santé, CHU Brest, France,
2Institut de Léprologie Appliquée, Dakar, Sénégal, Institut Pasteur, Dakar, Sénégal,
3Department of Immunology, Allergy and Infectious Diseases, St George Hospital, Kogarah, New South Wales, Australia

Keywords
Antiphospholipid syndrome, leprosy, β2 glycoprotein I, prothrombin

Summary
Anticardiolipin (ACA), anti-β2 glycoprotein I (β2-GPI), and anti-prothrombin antibodies of IgG and IgM classes were quantitated by enzyme-linked immunosorbent assays in 176 untreated leprosy patients across the histopathological spectrum. Positivity rates ranged from 21% (IgG ACA) to 30% (IgM anti-prothrombin) versus 4% in healthy controls (p < 10-2 to 10-3). Levels of IgM anti-β2-GPI and IgG ACA were significantly higher in lepromatous leprosy and multibacillary patient subgroups. IgG1 was the most common subclass reactive to both β2-GPI and prothrombin in selected high-titer leprosy sera, unlike antibodies from patients with the antiphospholipid syndrome (APS) largely restricted to IgG2. In leprosy patients, but not in the APS control group, there was no statistical correlation between ACA and anti-β2-GPI antibody levels. Likewise, a large fraction of anti-β2-GPI positive sera (36/45 and 28/44 for IgG and IgM, respectively) were unreactive in the standard ACA assay. Most assayed anti-β2-GPI antibodies from leprosy patients showed (i) ability to recognize both human and bovine β2-GPI immobilized on non-irradiated polystyrene plates, (ii) concentration-dependent inhibition of binding by cardiolipin, and (iii) relatively high avidity binding to fluid-phase β2-GPI, thereby differing from those found in APS. Finally, the location of the major epitopic region on the β2-GPI molecule targeted by autoantibodies was different in leprosy and APS, as assessed by direct binding to domain I- and V-deleted mutants and competition with the mouse monoclonal antibody 8C3, directed at domain I. Thus, leprosy-related antiphospholipid antibodies comprise persistent IgG and IgM anti-β2-GPI that differ from APS-related ones with respect to IgG subclass, avidity and epitope specificity, possibly reflecting distinct pathophysiological significance.

Introduction
Leprosy is a chronic infectious disease which presents a broad spectrum of manifestations reflecting the host immune response to Mycobacterium leprae, the causative intracellular pathogen. Type 1 T helper (Th1) cells are associated with tuberculoid leprosy (TT) in which a strong protective response reduces the bacterial load in patient tissues (paucibacillary). Th2 cells are preferentially associated with lepromatous leprosy (LL), which is characterized by polyclonal B cell activation and absence of M. leprae-specific T cell response, resulting in disseminated multibacillary disease, as well as development of hypergammaglobulinemia, immune complexities and a variety of autoantibodies (1, 2). Between the TT and LL poles, are immunologically unstable patients with intermediate symptoms corresponding to the borderline tuberculoid (BT), mid-borderline (BB) and borderline lepromatous (BL) forms (3).

Antiphospholipid antibodies (aPL) represent a heterogenous family of autoantibodies that are important clinically due to their association with recurrent arterial or venous thrombosis and pregnancy loss, condition termed antiphospholipid syndrome (APS) (4). This syndrome typically occurs in patients with autoimmune diseases, particularly systemic lupus erythematosus (SLE) and primary APS. The presence of aPL can also be reported in a variety of other settings, including malignancies, drug exposure and many infectious diseases (e.g. bacterial, viral and parasitic), conditions not associated with thrombophilia (5). Purification of the antibodies led to the discovery that most of those present in APS patients, and detected in first line assays as anticardiolipin antibodies (ACA) and lupus anticoagulants, do not bind directly to anionic phospholipids, but recognize the phospholipid-binding plasma proteins β2-glycoprotein I (β2-GPI) and prothrombin (4, 6, 7). This led us to develop protein antigen-based immunoassays, in the absence of phospholipids, in order to improve the detection of clinically relevant antibodies (8, 9). However, the schematic distinction between autoimmune-type ACA (β2-GPI-dependent, APS-associated) and infectious-type ACA (β2-GPI-independent, absence of thrombosis) has been recently challenged, with the report by Loizou et al. (10) that parvovirus B19-associated ACA require β2-GPI as a binding cofactor, similar to ACA found in SLE patients (11). With respect to leprosy, previous studies on the prevalence and clinical significance of different types of aPL have been few and controversial (2, 12-15). In particular, it is still unclear whether anti-β2-GPI antibodies are present and account for at least part of the “anticardiolipin” reactivity in leprosy patient sera.

Therefore the present work was designed to quantitate ACA, anti-β2-GPI and anti-prothrombin antibodies of IgG and IgM isotypes across the leprosy disease spectrum, and to compare anti-β2-GPI antibodies with those found in APS patients in terms of IgG subclass, avidity and epitope specificity.
Materials and Methods

Patients and Controls

The study population consisted of 176 untreated consecutive leprosy patients (103 males and 73 females; mean age 27.4 ± 16.2 years), newly registered at the “Institut de Léprologie Appliquée” (Dakar, Sénégal). The diagnostic criteria used were standard clinical signs and histological confirmation. Biopsies were obtained from active lesions, and the bacterial index (BI) determined microscopically. Patients, that were part of a previous therapeutic trial (16), were initially classified according to the Ridley-Jopling scale (3) as having LL, TT or borderline (including BB, BL and BT) leprosy. Forty-seven cases were grouped as undetermined, of whom 37 belonged to the paucibacillary group (BI = 0). None of the leprosy patients had clinical events suggesting APS. All patients were seronegative for the human immunodeficiency virus. Sera were obtained, after informed consent, from each patient before the initiation of anti-mycobacterial treatment, and kept at –20°C until use. Serial blood samples were obtained from 6 patients scoring positive for anti-β2GPI antibodies. Fifty healthy donors from the same area and with no known exposure to leprosy served as controls to determine the positivity threshold of antibody levels.

Forty-five patients with a history of venous or arterial thrombosis that fulfilled the classification criteria for definite APS (17) (26 primary and 19 secondary to SLE) were included in the positive aPL control group to compare antibody characteristics.

Materials

ELISA plates: γ-irradiated polystyrene (MaxiSorp) or plain polystyrene (PolySorp) (Nunc, RockIlde, Denmark). Human prothrombin (Diagnostica Stago, Asnières, France). Preparations of β2GPI: β2GPI isolated from human and bovine plasma (18); glyhis tagged domain deletion mutants, DM I-IV and II-V, purified as described (19). Cardiolipin from bovine heart in methanol, gelatin from cold water fish skin, Tween 20, and p-nitrophenyl phosphate (Sigma, L’Ile d’Abeau, France). Fetal calf serum (FCS) (Bio Whittaker, Verviers, Belgium). Alkaline phosphatase-conjugated affinity-purified goat anti-human IgG, γ chain specific, or IgM, μ chain specific (Zymed, San Francisco, CA).

Antiphospholipid Antibody Testing

The standard ELISA for ACA was performed using cardiolipin-coated plates (50 μg/ml in ethanol), 10% FCS in phosphate buffered saline (PBS) for saturation and serum sample dilution (1:100), and PBS washes.

In the CL β2GPI-ELISA, cardiolipin (0-50 μg/ml) was coated on PolySorp plates by solvent evaporation, then human or bovine β2GPI at 15 μg/ml in PBS was added for 2 h. Following PBS washing the plates were blocked with PBS/0.6% gelatin, then sequentially incubated with patient serum and second antibodies (18).

ELISAs for IgG and IgM anti-β2GPI and anti-prothrombin were performed using MaxiSorp plates coated with purified human proteins and PBS/Tween 0.1% as blocking agent and sample diluent, as previously described (9, 18).

For all ELISAs, (i) color development of plates was monitored until the absorbance of a positive control reached a predetermined value, (ii) the absorbance of control wells (sample-blank) was subtracted from the absorbance in the antigen-coated wells to account for nonspecific binding, and (iii) the cut-off points for positivity were set at the 96th percentile after testing 50-100 appropriate normal control sera.

IgG Subclass Analysis

This was achieved by the β2GPI- and prothrombin-ELISA as above, except for using working dilutions of mouse monoclonal antibodies (MAbs) to human IgG subclasses which gave a similar value for equal quantities of purified human IgG of each subclass. The following MAbs were used for IgG1 (HP6019), IgG2 (HP6014 mixed to HP6002), IgG3 (HP6047) and IgG4 (HP6025) determinations. Pooled sera from healthy donors provided the negative control for each assay.

Epitope Specificity and Avidity of Anti-β2GPI Antibodies

To localize possible immunodominant epitopes on β2GPI, highly positive serum samples from leprosy and APS patients were assayed using whole β2GPI, DM I-IV and II-V in parallel coated at 10 μg/ml on MaxiSorp plates.

The next steps were as above for the β2GPI-ELISA.

The ability of the anti-β2GPI MAb 8C3 (8) (shown here to bind domain I) to compete with the binding of patient samples in the β2GPI-ELISA was assessed as follows. β2GPI-coated plates were blocked and preincubated with 200 μg/ml of 8C3 for 15 min before adding for an additional 45 min an equal volume of patient serum diluted to twice the concentration exhibiting 50% of the maximum binding activity.

For estimating relative avidity of IgG anti-β2GPI from leprosy and APS patients, we measured the inhibition of binding to solid-phase β2GPI by fluid-phase β2GPI, as described (18).

Statistical Analysis

Comparisons of quantitative parameters were performed using Spearman’s rank correlation test, or non-parametric Mann-Whitney test. Comparisons of categorical parameters were performed using χ2 test or Fisher’s exact test when appropriate. Statistical significance was considered at p < 0.05.

Results

aPL Quantitation in Leprosy

Serum samples from 176 untreated patients with leprosy were screened for ACA, anti-β2GPI and anti-prothrombin antibodies of IgG and IgM isotypes. Table 1 summarizes positivity rate of each antibody type (21 to 30%), that was significantly higher than in the African control group (4% for all assays; p < 10^-2 to 10^-3). Likewise, the mean antibody levels in the study population, although rather low, were

<table>
<thead>
<tr>
<th>Nb positive (%)</th>
<th>ACA</th>
<th>anti-β2GPI</th>
<th>anti-prothrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any isotype</td>
<td>68 (39%)</td>
<td>69 (39%)</td>
<td>73 (41%)</td>
</tr>
<tr>
<td>IgG</td>
<td>37 (21%)</td>
<td>45 (26%)b</td>
<td>47 (27%)b</td>
</tr>
<tr>
<td>IgM</td>
<td>50 (28%)b</td>
<td>44 (25%)b</td>
<td>53 (30%)b</td>
</tr>
<tr>
<td>IgG alone</td>
<td>18</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>IgM alone</td>
<td>31</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>IgG + IgM</td>
<td>19</td>
<td>21</td>
<td>27</td>
</tr>
</tbody>
</table>

Patients versus healthy controls: a p < 10^-3; b p < 10^-3 and c p = 10^-3 by χ2 test.

Table 1 Prevalence and isotype of anticardiolipin (ACA), anti-β2GPI and anti-prothrombin antibodies in 176 patients with leprosy
significantly different from controls. As shown in Table 2, significant differences in mean antibody levels between leprosy subgroups were observed for IgM anti-β2-GPI and IgG ACA that were higher in lepromatous leprosy and multibacillary (BI of 1 to 6 representing logarithmic increases in bacterial numbers) patient groups.

As a whole, 133 out of 176 (75.5%) leprosy patients had at least one positive aPL detected while only 23 (13%) had the three of them and 30 (17%) had both anti-prothrombin and anti-β2-GPI antibodies. Finally, selected samples from leprosy patients with high-titer IgG anti-β2-GPI and/or anti-prothrombin were evaluated for their IgG subclass distribution, in parallel with positive sera from the APS control group (Table 3). In leprosy, IgG3 was the most common subclass reactive to both β2-GPI and prothrombin (89% and 91% positive, respectively) followed by IgG2 (33% and 45% positive, respectively). In contrast, the frequency of IgG1 positivity was 100% in the APS group while IgG1 and IgG3 marginally contributed to antibody activity, as expected from previous studies (9, 20).

**Relationship between ACA and Anti-β2-GPI Antibodies**

An intriguing finding in leprosy was the prominent discordance between the results of ACA and anti-β2-GPI antibodies, since 36 out of 45 (80%) sera positive for IgG anti-β2-GPI were missed by the standard ACA assay. The corresponding values for sera positive for IgM anti-β2-GPI were 28/44 (64%). Furthermore, in leprosy samples, there was no significant correlation between levels of IgG or IgM ACA with anti-β2-GPI antibodies of the same isotype, in sharp contrast with a high correlation for both IgG (rho = 0.81, p < 10⁻³) and IgM (rho = 0.66, p < 10⁻²) in 45 sera from the APS group (of whom 37 with anti-β2-GPI antibodies also scored ACA-positive).

For further evaluation of such discrepancies, we performed a “double-coating” assay on non-irradiated plates, the so-called CL/β2-GPI-ELISA (Fig. 1). Leprosy and APS sera differed with respect to (i) the ability to recognize β2-GPI on plain polystyrene in the absence of cardiolipin, and (ii) the consequence of cardiolipin addition in the first coating step. Most assayed anti-β2-GPI antibodies from leprosy patients were characterized by binding to both human and bovine β2-GPI on PolySorp plates and dose-dependent inhibition of binding in the presence of cardiolipin (Fig. 1A). The opposite was observed with APS sera (diluted 1:400 to avoid the influence of endogenous β2-GPI), i.e. no binding to PolySorp plates solely coated with a fixed amount of β2-GPI, and enhancement of binding when increasing cardiolipin concentration (Fig. 1B). Interestingly, all five leprosy sera shown in Fig. 1A did bind above background to cardiolipin alone (dashed line) in this assay using a gelatin buffer, despite being negative in the standard ACA assay performed in FCS. Such a behavior may reflect the presence of true ACA, associated with anti-β2-GPI antibodies in those samples.

The reason why anti-β2-GPI antibodies occurring in leprosy and APS have different plate requirement in order to be detected in a direct ELISA may relate to their avidity. Indeed, antigen density is likely to be higher on irradiated plates, leading to divalent binding of antibody and a threshold effect (7, 19). We thus compared relative avidity (defined by the dissociation constant, Kd) of IgG anti-β2-GPI from leprosy and APS patients in inhibition experiments using fluid-phase β2-GPI
Only slight inhibition was achieved using up to 20 μM (1 mg/ml) of β2 GPI in 8/22 APS samples and 1/14 leprosy sample. The latter sample, positive for both IgG and IgM ACA and anti-β2 GPI, also resembled APS samples in terms of epitope specificity (see below). The avidities of the IgG anti-β2 GPI from the remaining samples were significantly higher (p < 10^-4) in leprosy than in APS, the respective Kd averaging 6 × 10^-7 M and 9.5 × 10^-6 M.

**Epitope Specificity of Anti-β2 GPI Antibodies**

In order to compare the epitopes to which IgG and IgM anti-β2 GPI from leprosy and APS patients are directed, whole β2 GPI and two β2 GPI deletion mutants lacking either domain I (DM II-V) or domain V (DM I-IV) were directly immobilized side-by-side for testing 15 selected sera per group (Fig. 3). While all APS anti-β2 GPI antibodies recognized DM I-IV to a similar extent as the intact molecule, only two IgM bound to DM II-V above background. In contrast, the two mutants were almost equally recognized by leprosy antibodies, with the exception of one IgM (above-mentioned) directed at domain I as in APS, and of three samples that failed to bind to the mutants despite moderate to high binding to β2 GPI. As a further approach to study epitope location, the mouse MAb 8C3 that binds domain I (Fig. 3A) was used as competitor for patient antibody binding to solid-phase β2 GPI. The addition of MAb 8C3 led to marked inhibition of the anti-β2 GPI activity from APS sera, whereas inhibition was marginal or absent in leprosy, except for the domain I-specific IgM (Fig. 4).

Because aPL persistence is important to establish the diagnosis of APS (17), we performed a follow-up study over 2-year periods of anti-β2 GPI antibody levels in 30 serial samples from 6 leprosy patients with moderate to high titers on enrolment. Levels remained stable with time and no new isotype appeared for five patients, but they gradually decreased to normal in one (not shown).
Discussion

Despite a growing number of reports dealing with the links between aPL and infectious diseases, it is still unclear whether or not leprosy is causally related to the development of aPL and the APS. An important step has been the recognition that aPL are heterogeneous, i.e. directed against phospholipid-binding proteins in addition to phospholipids themselves (true aPL), and that only particular subsets are associated with a thrombotic diathesis (4, 6, 7, 11). Indeed, pathogenic aPL are largely directed to β2GPI, an abundant lipid-binding plasma protein whose crystal structure has recently been determined (21). Described as early as 1926, the Rubino test for leprosy (mostly positive in LL forms) which involves sedimentation of formalinized sheep erythrocytes by patient serum, has recently been recognized as a β2GPI-dependent aPL reaction (22). As a whole, reports using immunoassays (reviewed in 12) have been conflicting regarding aPL prevalence and isotype in leprosy, as well as the requirement of β2GPI in ACA binding. Fiallo et al. (14) suggested that IgG ACA in 140 multibacillary leprosy patients are mainly of the β2GPI-dependent type. Hojnik et al. (15) detected ACA in 98% of the 61 sera from LL patients, of which 32% showed decreased binding in the absence of bovine serum as a source of β2GPI, suggesting their β2GPI-dependency. However, the interpretation of such a modified ACA assay may be difficult, in particular due to the variable contribution to antibody binding made by endogenous β2GPI from patient serum. Using the more straightforward ELISA for anti-β2GPI antibodies, high antibody levels have been reported in 57% (almost all IgM) (12) and 18% (IgG being the sole isotype measured) (15) of LL patients. In contrast out of 35 LL patients, only one was positive for anti-β2GPI antibodies, despite ACA prevalence of 37% (13). In the present study, 39% of leprosy patients had IgG and/or IgM ACA and anti-β2GPI, the highest levels being measured in LL and multibacillary groups. These discrepancies may be ascribed to differences in the selection criteria and ethnic background of the patient populations.
for study, but above all to multiple methodological variables that affect aPL measurement.

In studies comparing the standard ACA test with the newer anti-β2-GPI ELISA, matters are complicated by the presence of true ACA solely reactive in the former and, most importantly, by the fact that the populations of anti-β2-GPI antibodies detected by the two assays are similar but not identical (7). The differences may be due to species specificity for human versus bovine β2-GPI, or to a variation of antigen presentation (18, 23). Indeed, the mode of presentation of β2-GPI in immunosassays (e.g. bound to cardiolipin or directly on irradiated plates) greatly influences its recognition by anti-β2-GPI antibodies, leading to considerable controversies as to their exact nature, i.e. anti-cryptic epitope versus low affinity antibodies (4, 7). In our leprosy patients, ACA and anti-β2-GPI antibodies appeared to be largely distinct antibody populations, as suggested by others in leprosy (15) and also in various infectious diseases (24). The most significant finding in the current study is that anti-β2-GPI antibodies from leprosy and APS patients exhibit very different properties with respect to recognition of the cardiolipin-β2-GPI complex, plate influence in anti-β2-GPI ELISA, and avidity for fluid-phase β2-GPI. It thus makes sense that different epitopes were targeted by these autoantibodies in the two patient groups. The mechanism of the observed competitive inhibition by MAb 8C3 of binding of anti-β2-GPI antibodies from APS patients probably involves direct masking of the corresponding epitope on domain I, rather than long-range effects through conformational changes in the MAb-bound β2-GPI molecule. Our data suggest that the immunodominant epitope(s) for anti-β2-GPI antibodies in APS patients are localized to the amino terminal domain (domain I) of β2-GPI in agreement with most (25-27), but not all (28) previous reports. In contrast, the dual recognition of β2-GPI deletion mutants observed with leprosy sera may reflect either discrete antibodies selective for individual domains, or antibodies that are cross-reactive between two or more domains (domains I-IV being highly homologous). That cardiolipin blocks the binding of anti-β2-GPI antibodies from leprosy patients (Fig. 1) thus cannot be equated with recognition of a single epitope located in the vicinity of the phospholipid binding site on domain V.

Autoantibodies have been widely reported in leprosy, but few of them appear related to autoimmune manifestations (2). Elbeialy et al. (13) found an association between the presence of ACA and dermatological signs, such as Raynaud’s phenomenon, skin ulcers, nodules and urticarial rash. The clinical significance, if any, of β2-GPI-dependent ACA or anti-β2-GPI antibodies remains largely unknown in leprosy. A recent case of aPL thrombotic syndrome in leprosy was initially misdiagnosed as Lucio’s phenomenon (29), although it cannot be excluded that APS reported as “secondary” to infections depend on coincidental events. We also measured anti-prothrombin antibodies by a direct ELISA because, in APS patients, they often occur in combination with anti-β2-GPI antibodies and share with them a number of immunochemical properties such as similar IgG subclass restriction (6, 9, 20). The mechanisms underlying the different subclass restriction of IgG anti-prothrombin and anti-β2-GPI between APS (IgG2) and leprosy (IgG3) patients are of interest. They may relate to the nature of the triggering antigens, as well as the microenvironment where these B cell responses take place, involving for example different types of T cells, cytokines and dendritic cells. In this respect, Hussain et al. (30) have noticed that disease progression in leprosy showed a significant correlation with selective increases in IgG1 and IgG3 responses to M. leprae, not due to selective polyclonal activation in these particular subclasses.

In conclusion, our findings corroborate the presence in leprosy of ACA, anti-β2-GPI and anti-prothrombin antibodies of IgG and IgM classes, but clinical complications such as thrombosis seem uncommon in these patients. In view of the different properties of anti-β2-GPI antibodies in the APS and leprosy groups, it is to be expected that only subpopulations of these antibodies have pathophysiologic significance. This raises an important issue for the necessary standardization and subsequent evaluation of anti-β2-GPI assays, and also to understand the role of β2-GPI in APS pathogenesis.

Acknowledgments

The authors thank Drs. JL Cartel and JP Lepers from the “Institut de Léprologie Appliquée, Dakar” for their support.

References


Received November 14, 2001 Accepted December 28, 2001